Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-bisphosphate-specific PH domain

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The signalling pathway leading, for example, to actin cytoskeletal reorganisation, secretion or superoxide generation involves phospholipase D (PLD)-catalysed hydrolysis of phosphatidylcholine to generate phosphatidic acid, which appears to mediate the messenger functions of this pathway [1–3]. Two PLD genes (*PLD1* and *PLD2*) with similar domain structures have been cloned and progress has been made in identifying the protein regulators of PLD1 activation, for example Arf and Rho family members [4,5]. The activities of both PLD isoforms are dependent on phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) and our sequence analysis suggested the presence of a pleckstrin homology (PH) domain in PLD1, although its absence has also been claimed [4]. Investigation of the inositide dependence showed that a bis-phosphorylated lipid with a vicinal pair of phosphates was required for PLD1 activity. Furthermore, PLD1 bound specifically and with high affinity to lipid surfaces containing PI(4,5)P_2 independently of the substrate phosphatidylcholine, suggesting a key role for the PH domain in PLD function. (GST–PLD1-PH) also bound specifically to supported lipid monolayers containing PI(4,5)P_2. Point mutations within the PLD1 PH domain inhibited enzyme activity, whereas deletion of the domain both inhibited enzyme activity and disrupted normal PLD1 localisation. Thus, the functional PH domain regulates PLD by mediating its interaction with polyphosphoinositide-containing membranes; this might also induce a conformational change, thereby regulating catalytic activity.

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Results and discussion

Phosphatidylcholine hydrolysis catalysed *in vitro* by PLD1b, the most commonly expressed PLD isoform, fused to GST (GST–PLD1b) is dependent on the presenceP of small G proteins and PI(4,5)P_2 [4,5]. Table 1 shows that, compared with phosphatidylserine, both natural and synthetic PI(4,5)P_2 and PI(3,4)P_2 were effective activators of PLD1b-catalysed phosphatidylcholine hydrolysis. PI(3,4,5)P_3, PI(3,5)P_2, phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol stimulated PLD activity only slightly. The same rank order of potency was obtained when the small GTP-binding protein Cdc42 or RhoA was substituted for Rac1 in the PLD assay (data not shown). Thus, synthetic and saturated inositol lipids were as effective in stimulating PLD as the natural (polyunsaturated) PI(4,5)P_2. Neither inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), the headgroup of PI(4,5)P_2, nor diacylglycerol could support PLD activity [6]. Chemical removal of the acyl chains from PI(4,5)P_2 generated glycerophosphatidylinositol 4,5-bisphosphate, which could not support PLD activity (data not shown).

gested that they contain no known polyphosphoinositide-binding domains [4]. Searches of the non-redundant sequence database held at the National Center for Biotechnology Information using PSI-BLAST, however, revealed

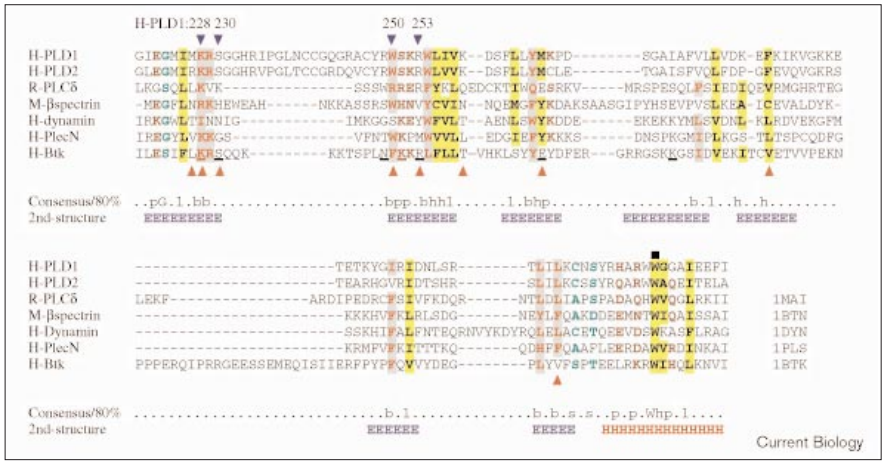
Table 1

Polyphosphoinositide dependence of PLD1b activity.

Activating phospholipid	Mean PLD activity (range) (dpm)
Phosphatidylserine (n)	1,607 (180)
Phosphatidylinositol (s)	2,661 (258)
sPtdIns3P	5,501 (163)
sPtdIns(3,4)P_2	17,842 (547)
sPtdIns(3,5)P_2	5,135 (352)
sPtdIns(4,5)P_2	16,762 (357)
sPtdIns(3,4,5)P_3	7,971 (901)
nPtdIns(4,5)P_2	19,571 (368)

Purified recombinant GST–PLD1 was assayed in the presence of Rac1 with vesicles containing phosphatidylethanolamine, phosphatidylcholine and the indicated natural (n) or synthetic (s) phospholipid [5]. Data are presented as mean decays per min (dpm) from duplicate experiments plus the range (indicated in parentheses). Similar results were obtained in several experiments.

Figure 1



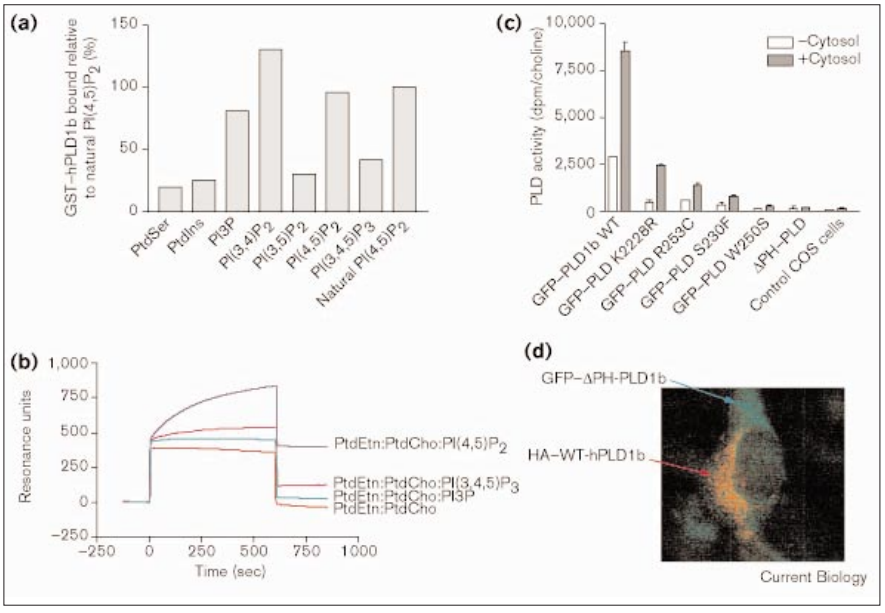
Alignment of PLD and Btk PH domains. Searches of protein sequences (ftp://ncbi.nlm.nih.gov/blast/db/nr) used the position-specific and iterative version of BLAST (PSI-BLAST) with no filters and an E-value inclusion threshold of E = 0.001. Alignment of PLD sequences against multiple alignments of known PH domains used the web-based resource (http://coot.embl-heidelberg.de/SMART/; [16]). The alignments were coloured after 80% consensus (calculated using http://bork.embl.heidelberg.de/cgi/consensus). Human (H) PLD1 and PLD2 PH domain sequences were compared with structure-based alignment of PH domain sequences of known high-resolution structure; sequence databases are held at NCBI. The carboxy-terminal sequence of the PI(3,4,5)P_3-binding protein [17] (amino acids 248–356) showed a significantly similar sequence in mouse PLD1b (amino acids 223–239) after four iterations ($E = 3 \times 10^{-4}$). The alignment is coloured after 80% consensus of the PH domain alignment available from SMART, β-strands (E) and α helices (H) are indicated beneath the alignment. The PDB codes are given following the alignment. Big (b) residues E, F, I, K, L, M, Q, R, W, Y (amino-acid single-letter notation) are shown red-on-grey; hydrophobic (h) residues A, C, F, I, L, M, V, W, Y and aliphatic (l) residues are shaded in yellow; polar (p) residues D, E, H, K, N, Q, R, S, T are in brown; and small (s) residues A, C, S, T, D, N, V, G, P are in green. A tryptophan, conserved in most PH domains, is indicated by the filled box. Residues substituted in patients with X-linked agammaglobulinaemia, are indicated by the red triangle. Residues in human PLD1 variants mutated in this study are indicated by a blue triangle. Residues in Btk that have been assigned to its Ins(1,3,4,5)P_4-binding site are underlined.

significant sequence similarity between known PH domains and a region of PLD1 and PLD2 located between the PX and catalytic domains [7]. An alignment of the PH domains of PLD1 and PLD2 with the PH domains of other proteins, including pleckstrin, phospholipase Cδ and Bruton's tyrosine kinase (Btk), is presented in Figure 1. Interestingly, amino acids assigned to the inositol-phosphate-binding site in the PH domain of Btk are conserved in the PH domain of PLD1 and PLD2 (Lys228, Ser230 and Arg253). Two recent theoretical studies using complementary methods have also suggested that PLD contains a PH domain [8,9]. As PH domains have been suggested to bind polyphosphoinositides specifically, and in view of the results shown in Table 1, it was considered that PLD1 might bind polyphosphoinositides.

Surface plasmon resonance (SPR) has been used to quantify the interaction of bacterial toxins with gangliosides [10], the binding of lipid vesicles to immobilised proteins [11] and the interaction of phosphoinositide-dependent kinase 1 (PDK1) with supported lipid monolayers [12]. The interaction of PLD with lipid monolayers comprising phosphatidylethanolamine, phosphatidylcholine and an activating lipid such as PI(4,5)P_2, was assessed by SPR. Efficiently coated hydrophobic surfaces bound very little bovine serum albumin and no free GST or GST–Janus-kinase-2 in the presence or absence of polyphosphoinositides (data not shown). A GST fusion containing the PH domain of the insulin receptor substrate 1 (GST–IRS1-PH) bound specifically to lipid surfaces containing natural or synthetic PI(4,5)P_2, but interacted weakly with surfaces containing phosphatidylserine or PI(3,4,5)P_3 (data not shown), as in previous studies of PI(4,5)P_2-dependent vesicle binding to antibody-immobilised GST–IRS1-PH [11]. Figure 2a shows that PLD1b interacted with PI(4,5)P_2 and PI(3,4)P_2, both of which could support enzyme activity. Conversely, phosphatidylserine, phosphatidylinositol, PI(3,5)P_2 and PI(3,4,5)P_3 did not support PLD activity and could not provide an efficient binding site for PLD. Although PLD bound to supported lipid monolayers containing PI3P, this lipid stimulated PLD activity only weakly (Table 1). Like PLD1, other PH domains, including those from phospholipase Cδ, diacylglycerol kinase-δ and β-adrenergic receptor kinase-1, exhibit a similar degree of promiscuity in their binding specificities by binding both PI(3,4)P_2 and PI(4,5)P_2 [13].

The binding of PLD1b to PI(3,4)P_2 and PI3P raises the possibility that PLD activity might be directly regulated by phosphatidylinositol 3-OH kinase (PI3K) lipid products. However, the cellular level of PI(4,5)P_2 exceeds agonist-stimulated levels of PI(3,4,5)P_3, PI(3,4)P_2 or PI3P. Furthermore, PLD activity has been shown to be agonist responsive in the absence of measurable PI3K activity [2]. It seems likely, therefore, that any role for PI3K in the regulation of PLD activity is indirect. In this regard, PI(3,4,5)P_3 has been shown to regulate the GTP status of Arf by interacting with the PH domain of the Arf guanine-nucleotide exchange factor ARNO [14].

Figure 2



PLD1 requires a functional PH domain for binding to supported lipid monolayers, enzyme activity and localisation. **(a)** GST–human PLD1b was specifically bound to supported lipid monolayer surfaces containing natural $PI(4,5)P_2$. Phosphatidylserine (PtdSer) or $PI(4,5)P_2$ (Lipid Products) or synthetic C_{16}-polyphosphoinositides (phosphatidylinositol (PI), PI3P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (Echelon Lipids) were presented to PLD in supported lipid monolayers of phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho), and similar results were obtained in two other experiments. **(b)** The PH domain of PLD1 bound specifically to $PI(4,5)P_2$-containing supported lipid monolayers. Purified recombinant GST–PH domain of PLD1b was injected across supported lipid monolayers containing phosphatidylethanolamine and phosphatidylcholine (red) plus the indicated polyphosphoinositide ($PI(4,5)P_2$, blue; $PI(3,4,5)P_3$, pink; PI3P, green). GST alone did not interact with these layers. Similar results were obtained in two other experiments. **(c)** Mutation of critical residues in the PH domain of PLD inhibited enzyme activity. The activity of mutant PLD constructs transfected and expressed in COS-1 cells was quantified. Data represent means from duplicate determinations plus range, and similar results were obtained in at least three other experiments. **(d)** Localisation of HA–WT-hPLD1b and GFP–ΔPH-hPLD1b in IIC9 cells. HA–WT-hPLD1b was detected with Texas Red-conjugated secondary antibody. The localisation of GFP–ΔPH-PLD1b (green) and HA–WT-hPLD1b (red) was assessed by confocal microscopy.

The interaction of PLD1b with supported lipid monolayers was not reduced in the absence of the phosphatidylcholine (data not shown) and therefore appeared to be independent of the active site. We determined that PLD had an apparent affinity of $2\,nM \pm 1\,nM$ for monolayers containing $PI(4,5)P_2$ in phosphatidylethanolamine. Analysis of these data, using BIA evaluation 3.0 software, suggested that the data fitted a simple 1 to 1 binding model ($\chi^2 < 0.25$). The high affinity of PLD1 for $PI(4,5)P_2$ is similar to the reported affinity of 1.6 nM for PDK1 binding to $PI(3,4,5)P_3$ [12]. A GST fusion protein of the PLD1b PH domain (amino acids 218–330 inclusive; GST–PLD-PH) was expressed and purified from *Escherichia coli*. Figure 2b shows that this domain bound to supported lipid monolayers containing $PI(4,5)P_2$, but interacted weakly with layers containing PI3P and PIP_3, indicating that the isolated PH domain and complete PLD1b have similar polyphosphoinositide-binding specificities. Recombinant GST did not interact with supported lipid monolayers in the presence or absence of polyphosphoinositides (data not shown). The interaction of PI3P with PLD1b, but not with the isolated PH domain, corresponds to the inability of this lipid to support catalytic activity, suggesting that its binding site is elsewhere in PLD.

Mutations of residues in the PH domain of Btk have been characterised in patients with X-linked agammaglobulinaemia (XLA). Of these, Phe25 to Ser or Arg28 to His in β strand 2, have been shown to reduce binding of inositol phosphate [15]. The corresponding residues in PLD1 and PLD2 are Trp250 and Arg253. Additionally, amino acids substituted in β strand 1 of Btk in XLA patients (Lys12 to Arg and Ser14 to Phe) are also conserved in PLD. In human PLD1 these residues correspond to Lys228 and Ser230. Thus, mutations analogous to those in the Btk PH domain were introduced into the PH domain of PLD1b. Lys228 was mutated to Arg, Ser230 to Phe, Phe250 to Ser and Arg253 to Cys. Wild-type and mutated PLD1b genes were expressed as green fluorescent protein (GFP) fusions in COS-1 cells that have low endogenous PLD activity (Figure 2c). The transfected PLD activity was determined in cell lysates, using HL60 cell cytosol as a source of necessary protein activators. Mutation of the conserved residues within the PH domain of PLD1 dramatically reduced basal and stimulated enzyme activity (Figure 2c). Comparable results were obtained with PLD mutants tagged with haemagglutinin (HA) at the amino terminus (data not shown). Mutation of a potentially non-critical residue within the PH domain did not affect PLD activity (data not shown). Finally, deletion of the PH domain from PLD1b (GFP–ΔPH-PLD1b) generated an inactive PLD.

The localisation of wild-type HA-tagged PLD1b (HA–WT-PLD1b) and the GFP-tagged PLD1b with a deleted PH domain (GFP–ΔPH-PLD1b) was examined following transient transfection. The two PLD1b constructs did not co-localise in IIC9 fibroblasts (Figure 2d).

Wild-type PLD1b localised to an endosomal or lysosmal compartment, consistent with our previous observations in COS-1 and RBL-2H3 cells [3]. In contrast, GFP–ΔPH-PLD1b assumed a punctate distribution throughout the cell; however, it was not possible to define its location. Mutation of single amino acids in the PH domain of PLD1 (as above) did not affect localisation (data not shown). These observations demonstrate a critical role for the PH domain in regulating the subcellular localisation of PLD1b.

Our results show that human PLD1 contains a functional PH domain. The polyphosphoinositide dependence of PLD1b activity can be ascribed to the ability of the enzyme to physically interact with lipids via the PH domain. Point mutations within the PH domain inhibited PLD activity, while deletion of the PH domain both inhibited enzyme activity and distorted the normal subcellular localisation of PLD1b, emphasising the essential role of the PH domain in the control of both enzyme activity and subcellular localisation. It has been reported previously that PH domains promote both membrane interaction and increased enzymatic activity of signalling proteins [14]. Finally, our results suggest that the interaction of PLD with $PI(4,5)P_2$-bearing membranes may be a key event leading to the formation of a multicomponent complex of lipids and regulatory proteins, resulting in phosphatidylcholine hydrolysis.

Materials and methods

Expression and purification of GST–PLD1b

GST–PLD1b was expressed in insect cells, purified and assayed as previously described [5]. GST–IRS1-(PH) (a generous gift of G. Panayotou, Ludwig Institute, London), free GST and GST–PLD1b-PH were expressed in *E. coli* and purified by glutathione–Sepharose affinity chromatography and gel filtration. GST–Janus-kinase-2 was from Santa Cruz Biologicals.

Surface plasmon resonance analysis of the binding of PLD1b to supported lipid monolayers

Surface plasmon resonance was performed using a Biacore 2000 and hydrophobic HPA chips (Biacore UK). Supported lipid monolayers were formed following injection of 2% octylglucoside into 300 μM extruded unilamellar vesicles (phosphatidylethanolamine 85mol%; phosphatidylcholine 5mol%; $PI(4,5)P_2$ or acidic lipid as indicated 10mol%) across the HPA surface at 3 μl/min. Surfaces were subjected to a high flow rate, several injections of 100 mM HCl and 10 μg BSA [10]. Specificity experiments utilised GST–PLD1b (50 nM) or GST–PLD1-PH (100 nM) at a flow rate of 5 μl/min, while the affinity of PLD1b for the surface was determined at 1–25 nM at flow rates of 10, 20 and 30 μl/min.

Expression of GFP–PLD1b mutants in COS-1 cells

Human PLD1b (hPLD1b) was subcloned into the GFP vector eGFP to produce the amino-terminal fusion protein GFP–hPLD1b [3]. The PLD point mutants (K228R, S230F, W250S, R253C) were obtained by PCR site-directed mutagenesis and subcloned back into eGFP containing wild-type hPLD1b. GFP–PLD1b-PH was generated by Expand™ High Fidelity PCR system (Roche Diagnostics). COS-1 cells (1×10^6) were transiently transfected with 5 μg DNA constructs in the presence of polyethylenimine, lysed after 24 h, and PLD activity determined in the presence and absence of HL60 cytosol [5].

Expression of HA–WT-PLD1 and GFP–ΔPH-PLD1b in IIC9 fibroblasts

IIC9 fibroblasts were transfected with 200 ng HA–WT-PLD1 DNA and GFP–ΔPH-PLD1b in the presence of 1.5 μl Fugene™. After 24 h cells were fixed, permeabilised and incubated with primary antibody (12CA5, mouse anti-HA) and goat anti-mouse IgG labelled with Texas Red. Fluorescence was detected using a deconvoluting confocal microscope.

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